PB86-157971

Phenylmethylsulfonyl Fluoride Protects Rats from Mipafox-Induced Delayed Neuropathy

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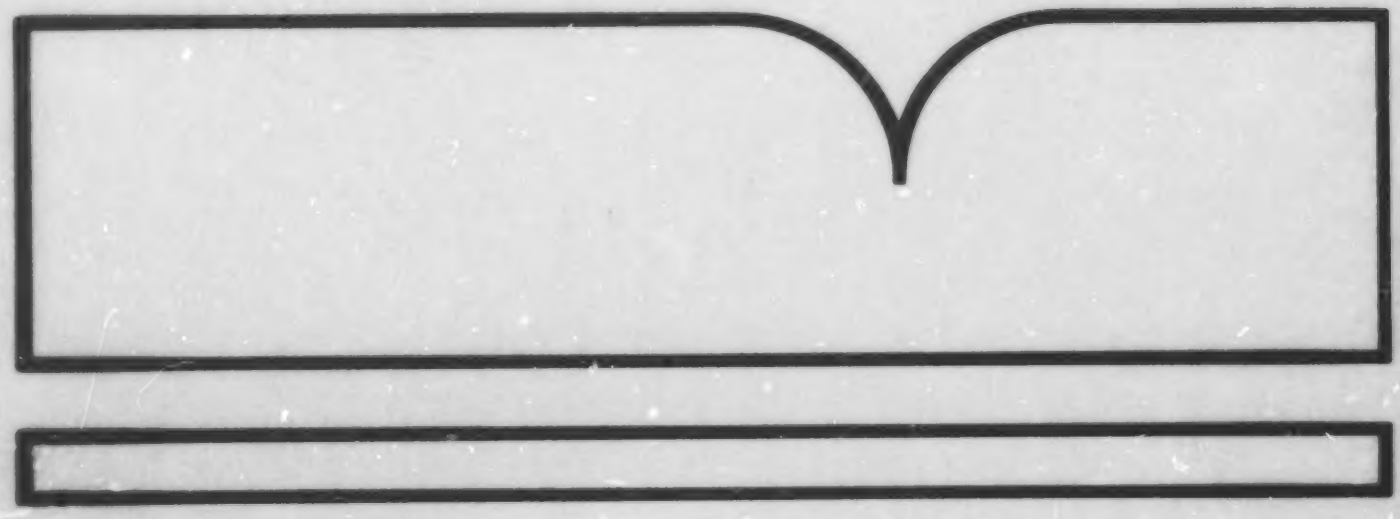
Research Triangle Park, NC

1985



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1. REPORT NO.	2.	3. R
EPA/600/J-85/281		PB86-157971

4. TITLE AND SUBTITLE	5. REPORT DATE
Phenylmethylsulfonyl Fluoride Protects Rats From Mipafox-Induced Delayed Neuropathy (Journal Version)	1985
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Bellina Veronesi and Stephanie Padilla	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Neurotoxicology Division Health Effects Research Laboratory US Environmental Protection Agency Research Triangle Park, NC 27711	ACAE1A
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Health Effects Research Laboratory Office of Research and Development US Environmental Protection Agency Research Triangle Park, NC 27711	14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Toxicology and Applied Pharmacology 81, 258-264 (1985) November

16. ABSTRACT

Initiation of organophosphorus-induced delayed neuropathy (OPIDN) is thought to consist of two molecular events involving the phosphorylation of the target enzyme, neurotoxic esterase or neuropathy target enzyme (NTE), and a subsequent "aging" reaction which transforms the inhibited NTE into a charged moiety critical to the neuropathic process. Compounds that inhibit NTE but cannot age because of their chemical structure abort this two-stage initiation process, and when administered before a neurotoxic organophosphorus compound (OP), protect against the neuropathy by blocking NTE's active site (Johnson, 1970). In support of this, we report that prior exposure to a non-aging NTE inhibitor, phenylmethylsulfonyl fluoride (PMSF), protects rats from neurological damage after subsequent expsoure to a neurotoxic OP, Mipafox. Adult, male Long Evans rats were exposed to either PMSF (250mg/kg, sc) or to Mipafox (15 mg/kg,ip) and a time-course of brain NTE inhibition and recovery was defined. A separate group fp PMSF-treated rats was exposed to Mipafox when brain NTE inhibition was 87.7 + 2.3%. Conversely, another group of rats, pretreated with Mipafox, was dosed with PMSF when NTE inhibition was 90.2 + 0.8%. A third group of animals, treated with PMSF was exposed to Mipafox 14 days later, when NTE activity had recovered to within 10 + 4.2% of control levels. Histopathological survey (14-21 days post-exposure) indicated severe cervical cord damage (damage score >3) in the following frequencies: PMSF=0%; Mipafox=85%; PMSF-4hr-Mipafox=0%; Mipafox-4hr-PMSF=100%; PMSF-14d-Mipafox=75%; controls=0%. These data indicate that PMSF pretreatment protects rats against Mipafox-induced neurological damage and that the timing of administration and order of presentation is critical to this protection.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	8
	20. SECURITY CLASS (This page)	22. PRICE
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JOURNAL ARTICLE

TOXICOLOGY AND APPLIED PHARMACOLOGY 81, 258–264 (1985)

Phenylmethylsulfonyl Fluoride Protects Rats from Mipafox-Induced Delayed Neuropathy

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Received March 12, 1985; accepted June 14, 1985

Phenylmethylsulfonyl Fluoride Protects Rats from Mipafox-Induced Delayed Neuropathy. VERONESI, B. AND PADILLA, S. (1985). *Toxicol. Appl. Pharmacol.* 81, 258–264. Initiation of organophosphorus-induced delayed neuropathy (OPIDN) is thought to consist of two molecular events involving the phosphorylation of the target enzyme, neurotoxic esterase, or neuropathy target enzyme (NTE), and a subsequent "aging" reaction which transforms the inhibited NTE into a charged moiety critical to the neuropathic process. Compounds that inhibit NTE but cannot age because of their chemical structure abort this two-stage initiation process, and when administered before a neurotoxic organophosphorus compound (OP), protect against the neuropathy by blocking NTE's active site (Johnson, 1970). In support of this, we report that prior exposure to a nonaging NTE inhibitor, phenylmethylsulfonyl fluoride (PMSF), protects rats from neurological damage after subsequent exposure to a neurotoxic OP, Mipafox. Adult, male, Long Evans rats were exposed to either PMSF (250 mg/kg, sc) or to Mipafox (15 mg/kg, ip) and a time course of brain NTE inhibition and recovery was defined. A separate group of PMSF-treated rats was exposed to Mipafox when brain NTE inhibition was 87.7 ± 2.3%. Conversely, another group of rats, pretreated with Mipafox, was dosed with PMSF when NTE inhibition was 90.2 ± 0.8%. A third group of animals, treated with PMSF, was exposed to Mipafox 14 days later, when NTE activity had recovered to within 10 ± 4.2% of control amounts. Histopathological survey (14 to 21 days post-exposure) indicated severe cervical cord damage (damage score ≥3) in the following frequencies: PMSF, 0%; Mipafox, 85%; PMSF–4 hr–Mipafox, 0%; Mipafox–4 hr–PMSF, 100%; PMSF–14 days–Mipafox, 75%; controls, 0%. These data indicate that PMSF pretreatment protects rats against Mipafox-induced neurological damage and that the timing of administration and order of presentation are critical to this protection. These results support the hypothesis that the initiation of OPIDN is a multistage event involving inhibition and aging, and that these stages are experimentally separable. © 1985 Academic Press, Inc.

Acute exposure to many, but not all, organophosphorus (OP) esters produces a delayed neuropathy known as OPIDN after a quiescent period of 8 to 14 days (Cavanagh, 1964; Abou-Donia, 1981). This neuropathy follows a "dying-back" pattern of damage, originating in the distal extremities and involving, with time, more proximal lengths of the affected large-diameter nerve fibers. Initiation of this damage is purported to involve two separate molecular events: the OP's inhibition (i.e., phosphorylation) of the target protein, neurotoxic esterase, or neuropathy target enzyme (NTE) by at least 70%, and the loss of a side chain group from the inhibited enzyme–inhibitor complex (i.e., "aging") to produce a charged, monosubstituted phosphoric acid group attached to the NTE (Clothier and Johnson, 1979, 1980; Williams, 1983). NTE's inhibition can be conveniently monitored by an *in vitro* assay which defines the loss of catalytic activity (Johnson, 1969, 1977). It is the subsequent aging reaction, however, that is a prerequisite for the degeneration of nerve fi-

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0041-008X/85 $3.00

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bers by some still unexplained mechanism (Johnson, 1974, 1984). Various compounds (e.g., certain phosphinates, carbamates, and sulfonates) are able to inhibit NTE over the critical 70%, but because of their chemical structure cannot age and are therefore non-neurotoxic (Johnson, 1970, 1974). Administration of such compounds prior to treatment with an ageable OP blocks NTE's active site and actually protects the organism against OPIDN (Johnson and Lauwerys, 1969; Johnson, 1970, 1974; Baker *et al.*, 1980; Carrington and Abou-Donia, 1983; Caroldi *et al.*, 1984). However, if administration of the neurotoxic OP is delayed until ≥70% of NTE activity becomes available for phosphorylation via resynthesis, the animal is again susceptible to OPIDN upon exposure to a neurotoxic OP (Johnson, 1970, 1974).

In recent studies, we have demonstrated that the rat, previously thought to be insensitive to OPIDN because of its resistance to ataxia, is vulnerable to the central and peripheral neuropathic damage resulting from acute or multiple doses of tri-*ortho*-cresyl phosphate (TOCP) (Veronesi, 1984a). Furthermore, the rat's sensitivity to TOCP-induced neurological damage can be manipulated by metabolic inhibitors such as piperonyl butoxide (Veronesi, 1984b). In the present study, the rodent model of OPIDN was used to assess if prior exposure to a nonaging sulfonate (i.e., phenylmethylsulfonyl fluoride, PMSF) protects against subsequent Mipafox-induced neuropathic damage.

METHODS

Animals. Long Evans 60-day-old male rats (Blue Spruce Laboratories, NY) were housed in pairs on sterile pine bedding and allowed free access to feed (Purina Rat Chow) and water. The animals were housed under a lighting schedule of 12-hr light/12-hr dark at 22°C and 50 to 60% relative humidity.

Chemicals. PMSF and atropine sulfate were obtained commercially (Sigma Chemical Co., St. Louis, MO) as was paraoxon (*O,O*-diethyl *O*,4-nitrophenyl phosphate) (Aldrich Chemical Co., Milwaukee, WI). *N,N'*-Diisopropylphosphorodiamidic fluoride (Mipafox) and phenyl valerate, the substrate for the NTE assay, were synthesized by S. Soliman (Alexandria, Egypt).

Animal dosing and treatment groups. Animals for biochemistry and pathology were dosed contemporaneously and divided into the following treatment groups: (A) PMSF (250 mg/kg) dissolved in 95% ethanol was administered subcutaneously (sc) in the nape of the neck. (B) Mipafox (15 mg/kg) dissolved in saline was injected intraperitoneally (ip). (C) PMSF (250 mg/kg) was followed 4 hr later by Mipafox (15 mg/kg): PMSF–4 hr–Mipafox. (D) Mipafox (15 mg/kg) was followed 4 hr later by PMSF (250 mg/kg): Mipafox–4 hr–PMSF. (E) PMSF (250 mg/kg) followed 14 days later by Mipafox (15 mg/kg): PMSF–14 days–Mipafox. (F) Controls: Group A was given 95% ethanol; Groups B and D were given saline (1 ml/kg, ip) and atropine (30 mg/kg in saline, ip). Groups C and E were given 95% ethanol followed by saline + atropine. All rats treated with Mipafox were prophylactically treated with atropine 20 min pre- and 4 hr post-dosing. The animals' conditions were recorded twice a day for 72 hr post-treatment.

NTE assay. Rats ($n = 3$ to 5) from Groups A and B with appropriate controls were decapitated 1, 4, 24, 48, and 72 hr after PMSF or Mipafox administration. Additional animals treated with PMSF were assayed 14 days post-exposure. The brain, including the cerebellum, was removed, weighed, and immediately placed in 5 vol (w/v) of ice-cold buffer (50 mM Tris/HCl, 0.2 mM EDTA, pH 8.0 at 24°C), and homogenized at 4°C for 15 sec (speed 6) with a Polytron apparatus (Brinkman Instruments, Westbury, NY). The homogenates were centrifuged to remove cell debris (4°C at 9000g_{av} for 15 min), obtaining a supernatant fraction less turbid than the original homogenate. With this supernatant fraction, NTE activity was assayed within 1 hr postmortem. NTE activity was defined as the difference in phenyl valerate-hydrolyzing activity between paired samples in the presence of either paraoxon or paraoxon plus Mipafox (Johnson, 1977; Soliman *et al.*, 1982). Duplicate tubes were incubated for 20 min at 37°C containing buffer with either (A) paraoxon (final concentration, 40 μM) or (B) paraoxon and Mipafox (final concentrations, 40 and 50 μM, respectively) in a final volume of 3 ml. Following the preincubation, phenyl valerate was added to start the enzymatic reaction. After 10 min at 37°C, the reaction was terminated (2.0 ml of 1.0% sodium dodecyl sulfate with 0.025% 4-amino antipyrine) and the color reaction was completed [1.0 ml of 0.4% $K_3Fe(CN)_6$]. Absorbance was read at 510 nm, and NTE activity, calculated as the difference in activity between tubes (A) and (B), was expressed as specific activity. Tissue protein concentrations were measured according to the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Tissue preparation for neuropathology. Rats from treatment Groups A to F ($n = 5$ to 13) were killed 14 to 21 days post-exposure by whole-body perfusion with Sorenson's buffered 5% glutaraldehyde. The upper (C2) cervical-level spinal cord was removed, divided in three parts, post-

fixed in Dalton's 2% osmium tetroxide, dehydrated in ethanol, cleared in propylene oxide, and embedded in an epoxy resin. The cervical cord tissue was semithinned (1.0 μm) and stained with 1% toluidine blue-o. Thin (60 to 70 nm) sections for electron microscopic examination were stained with uranyl acetate and lead citrate.

Cervical cord damage scoring. The fasciculus gracilis of the cervical cord was ranked on a scale of 0 to 4 according to the severity of axonal damage. Sections were scored by the following criteria: 0, no fiber degeneration or myelin debris noted; 1, individual or small groups (2 to 3) of swollen/degenerated axons present; 2, narrow margin of degeneration along the dorsal column's ridge; 3, wider and more lateral band of degeneration noted along the fasciculus; and 4, funnel-shaped pattern of fascicular degeneration. All three semithin sections were examined from each animal, read blind, and individually scored. All scores were averaged, and these values were pooled according to dosage group to yield mean damage scores.

RESULTS

Animal Response to Treatment

Group A. PMSF, 250 mg/kg. Animals exposed to PMSF became incapacitated 1 hr post-dosing and remained so for 48 hr, after which time they returned to normal activity and feeding habits. This debilitation appeared related to the ethanol vehicle as it was noted in the ethanol control animals (see below). Severe necrotic skin lesions were observed at the injection site. No mortality occurred in this group.

Group B. Mipafox, 15 mg/kg. Immediately after treatment, mild tremors occurred in 60% of the animals. Atropine treatment (4 hr post-exposure) alleviated these tremors. The animals remained agitated and hypersensitive to loud sounds for another 24 hr. Diarrhea, noted in 30% of the rats, dissipated by 72 hr post-exposure. No mortality occurred in this group.

Group C. PMSF–4 hr–Mipafox. Animals were immobile 30 min post-exposure and remained so for 48 hr. Mipafox-associated anticholinesterase signs did not appear. This group experienced the highest (50%) mortality.

Group D. Mipafox–4 hr–PMSF. Mipafox-associated tremors were noted immediately, but were abolished upon subsequent PMSF exposure. This latter treatment immobilized the animals for 48 hr. A 25% mortality occurred.

Group E. PMSF–14 days–Mipafox. PMSF exposure resulted in complete incapacitation as described above. Mipafox treatment 14 days later produced the typical parasympathetic signs (i.e., tremors, diarrhea) described earlier. No mortality occurred.

Group F. Controls. Animals exposed to the 95% ethanol vehicle appeared debilitated immediately after treatment, but recovered the next day. Severe skin lesions developed at the point of ethanol injection. Atropine controls appeared lethargic 30 min after treatment and remained so for 24 hr.

Time Course of NTE Activity

Figure 1 shows the inhibition–recovery profile of brain NTE in rats exposed to either

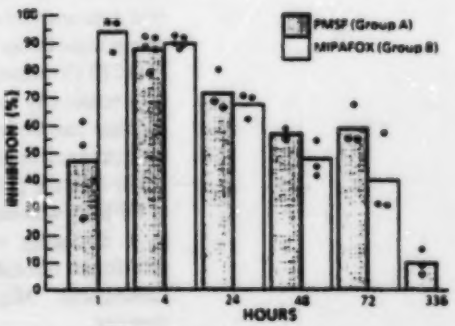

FIG. 1. Time course of brain NTE inhibition after PMSF (Group A) or Mipafox (Group B) exposure. Treated and appropriate controls were killed at the time points indicated on the horizontal axis. Brain NTE activity was expressed as percentage inhibition compared to controls. The bars represent the mean (n = 3 to 5) NTE inhibition, while the filled circles indicate the NTE inhibition values for each animal sampled at that time. NTE values for the ethanol controls were 1 hr, 14.2 ± 1.1; 4 hr, 12.4 ± 0.2; 24 hr, 10.3 ± 0.5; 48 hr, 12.1 ± 0.3; 72 hr, 11.9 ± 0.2; and 14 days, 14.4 ± 1.4 nM phenyl valerate hydrolyzed min^{-1} mg $protein^{-1}$. For the saline/atropine controls: 1 hr, 10.9 ± 0.6; 4 hr, 10.9 ± 0.4; 24 hr, 11.5 ± 0.2; 48 hr, 10.3 ± 0.3; and 72 hr, 9.9 ± 0.9 nM phenyl valerate hydrolyzed min^{-1} mg $protein^{-1}$.

PMSF (Group A) or Mipafox (Group B). At 1 hr post-treatment, NTE was inhibited by 46.8 ± 10.6% in the PMSF-treated animals compared to 94.2 ± 3.7% in the Mipafox-treated rats. In both Groups A and B near maximal NTE inhibition occurred at 4 hr post-dosing, a time point chosen for our combination treatments (Groups C and D). The recovery profiles of brain NTE activity in Groups A and B were similar with the activity returning to approximately 50% of control values by 2 days post-dosing.

Histopathology

Preferential axonal degeneration of fasciculus gracilis of the cervical cord was seen in Groups B, D, and E. In these animals, degeneration consisted of hyaline bodies, myelin ellipsoids, and giant swollen axons (Fig. 2). Ultrastructurally, these axonal swellings were either distended with accumulations of interlaced smooth endoplasmic reticulum or formed by an intraaxonal vacuole which compressed to one side of the axolemma a thin rim of membrane-bound axoplasm. Both types of coexisting axonal changes were used to derive the damage scores for the fasciculus gracilis.

Rats exposed to PMSF (Groups A, C, and E) displayed myelin droplets throughout the cervical funiculi and those from Group D (Mipafox–4 hr–PMSF) and Group E (PMSF–14 days–Mipafox) contained both myelin droplets and axonal swellings.

Scoring of Cord Damage

The data are presented as a composite scattergram/bar graph (Fig. 3). The damage was ranked 0 to 4 depending on the amount and location of axonal damage. The mean score from Group A (1.09 ± 0.33) indicated a marginal baseline of PMSF-associated damage which consisted of myelin droplets. In contrast, a mean damage score of 3.08 ± 0.01 de-

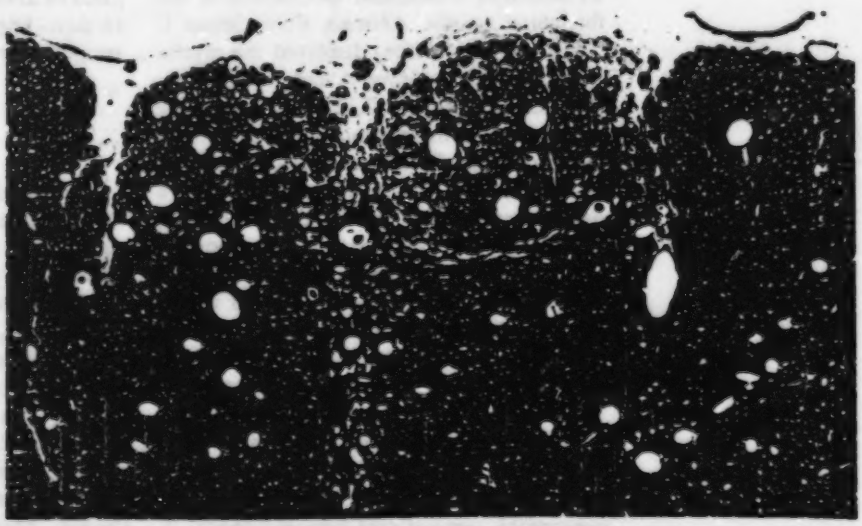

FIG. 2. The cervical spinal cord of animals displayed swollen axons (arrow), vacuolated axons, and myelin debris in the fasciculus gracilis. The distribution and severity of damage in this section merited a damage score of 3. Tissue was taken from a Mipafox-treated rat (Group B). Semithin epoxy section; toluidine-blue stained; ×450.

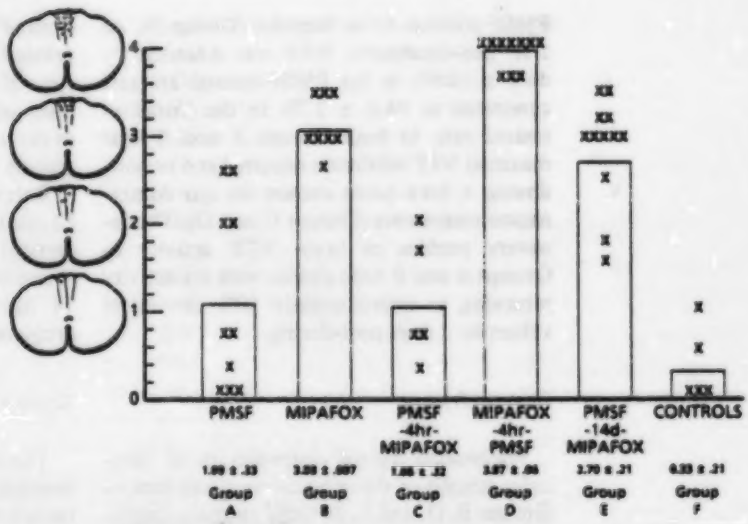

FIG. 3. Semiquantification of cervical cord damage is represented in both scattergram and bar graph form and the $\bar{x} \pm$ SE for each group is noted. Neuropathy was scored 0 to 4, depending on the amount and location of axonal damage. Cord involvement is depicted diagramatically along the vertical axis.

rived from the Mipafox-treated rats (Group B) described widespread destruction of the fasciculus gracilis. Animals from Group C (PMSF–4 hr–Mipafox) displayed the myelin droplets noted in Group A and negligible axonal damage (1.06 ± 0.32). Alternating the presentation of these compounds in Group D (Mipafox–4 hr–PMSF) exacerbated the cervical pathology, which was evidenced by a damage score of 3.87 ± 0.06. In test Group E (PMSF–14 days–Mipafox), Mipafox administration to PMSF-pretreated rats was withheld until NTE activity had recovered to within 10% of control amounts. This regimen yielded cervical cord damage (2.70 ± 0.21) similar to that seen in rats treated only with Mipafox.

As in previous studies which used a semiquantified scoring of spinal cord damage (Padilla and Veronesi, 1985), we elected to use a damage score of ≥3 to indicate definitive neuropathic cervical cord damage (see Fig. 2). With that endpoint, neuropathy was noted in the following frequency: Group A (PMSF), 0%; Group B (Mipafox), 85%; Group C (PMSF–4 hr–Mipafox), 0%; Group D (Mipafox–4 hr–PMSF), 100%; Group E (PMSF–14 days–Mipafox), 75%; Group F (ethanol–atropine controls), 0%.

DISCUSSION

This experiment not only demonstrates that PMSF pretreatment protects rats against the neurological damage of OPIDN, but helps to differentiate the initial molecular events underlying the neuropathy. Because of its direct (i.e., not requiring prior metabolic activation), rapid inhibition of NTE and its ability to age due to the presence of a labile R-NH-P bond, Mipafox was selected as the neurotoxic OP. PMSF was chosen because of its precedential use as a nonaging, NTE-inhibiting blocking agent in other protection experiments with the chicken (Johnson, 1974; Carrington and Abou-Donia, 1983; Caroldi *et al.*, 1984) and the cat (Baker *et al.*, 1980) model of OPIDN. The presence or absence of neuropathy in rats

dosed with either Mipafox or PMSF supports the principle that aging rather than inhibition is the critical event in precipitating OPIDN, an observation noted in other species (Johnson and Lauwerys, 1969; Johnson, 1970, 1974; Baker *et al.*, 1980; Carrington and Abou-Donia, 1983). PMSF's protection against subsequent neurotoxic challenge is thought to result from its "masking" of NTE's active site, which manifests as inhibition and yet forestalls the neuropathic consequences (Johnson, 1970, 1974; Carrington and Abou-Donia, 1983). The mere presence of PMSF, however, is not sufficient to protect since alternating the order of exposure (i.e., Mipafox–4 hr–PMSF), allows Mipafox to inhibit and age the NTE and initiate the neuropathy. The neurological damage seen in animals pretreated with PMSF and exposed 14 days later to Mipafox demonstrates that the effectiveness of PMSF's protection is relative to the recovery of NTE activity. Taken in concert, these data emphasize that the timing and order of presentation of ageable and nonageable compounds are critical to the protection phenomenon, and that modification of these parameters produces dramatically different neuropathic results.

The significantly higher damage score of the Mipafox–4 hr–PMSF-treated rats over those dosed only with Mipafox suggests that PMSF compounds Mipafox-induced damage, probably through its antiprotease activities (Barret, 1980). We stress that the PMSF-related damage (i.e., myelin droplets) was qualitatively and topographically different from that induced by Mipafox alone, and propose that it was related to the rather high doses of PMSF used in this experiment (8 to 10 times that used to protect chickens). Although much lower concentrations of the sulfonyl fluoride (approximately 150 mg/kg) will inhibit NTE ≥80% in Sprague–Dawley rats (R. Richardson, personal communication), we hoped to sustain a high level of PMSF-induced NTE inhibition throughout the course of treatment, eliminating the necessity of redosing the animals. The demonstration of PMSF-related nerve damage is not unique to this experiment, being indicated in an earlier study which used the sulfonyl fluoride as a protective agent against diisopropylphosphorofluoridate (DFP) neuropathy in cats (Baker *et al.*, 1980). Using electrophysiological endpoints, these investigators reported that the ability of soleus alpha-motor nerve terminals to generate stimulus bound repetitive action potentials was mildly diminished in PMSF-treated cats compared to controls.

The demonstration that PMSF protects rats against a challenge dose of a neuropathic organophosphate suggests that the initial molecular events of OPIDN are common to all tested species and underscores the critical role of the inhibited and aged NTE for the neuropathic process.

ACKNOWLEDGMENTS

The authors thank Dale Newland, Al Inman, and Donald Lyerly for their excellent technical assistance, and Ava Hinton for her preparation of this manuscript. This manuscript has been reviewed by the Health Effects Research Laboratory, USEPA, and approved for publication. Mention of trade names and commercial products does not constitute endorsement of recommendation for use.

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